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The *STUD* Gene Is Required for Male-Specific Cytokinesis after Telophase II of Meiosis in *Arabidopsis thaliana*

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During male meiosis in wild-type *Arabidopsis* the pollen mother cell (PMC) undergoes two meiotic nuclear divisions in the absence of cell division. Only after telophase II is a wall formed which partitions the PMC into four microspores. Each microspore undergoes two subsequent mitotic divisions to produce one vegetative cell and two sperm cells in the mature pollen grain. In this paper we describe the isolation and the phenotypic characterization of mutations in the *STUD* (*STD*) gene, which is specifically required for male-specific cytokinesis after telophase II of meiosis. Although the male meiotic nuclear divisions are normal in *std* mutant plants, no walls are formed resulting in a tetranucleate microspore. Despite the absence of cell division in the PMC, postmeiotic development in the coenocytic microspore proceeds relatively normally, resulting in the formation of large pollen grains which contain four vegetative nuclei and up to eight sperm cells. Interestingly, these enlarged pollen grains which contain multiple vegetative nuclei and extra sperm cells behave as single male gametophytes, producing only single pollen tubes and resulting in partial male fertility in *std* mutant plants. Characterization of the process of pollen development and pollen function in *std* mutants thus reveals two different types of developmental regulation. Each of the four nuclei found in a *std* microspore following meiosis is capable of independently undergoing the complete mitotic cell division (including cytokinesis) which the single nucleus of a wild-type microspore would normally undertake. The ability of the four meiotic products to independently continue through mitosis does not depend on their division into separate cells, but is controlled by some subcellular component found within the coenocytic micropsore. By contrast, the mature *std* pollen grain functions as a unit and produces only a single pollen tube despite the presence of multiple nuclei within the vegetative cell, suggesting that this process is controlled at the cellular level independently of the extra subcellular components.

INTRODUCTION

Cells reproduce by cell division which is composed of a nuclear division (mitosis) followed by a cytoplasmic division (cytokinesis). In most animal cells cytokinesis is accomplished by the contraction of a thin ring composed of actin and myosin II filaments. In contrast plant cells are enclosed in a rigid cell wall and cytoplasm is partitioned by construction of a new cell wall inside the dividing cell. The future division plane is marked by the preprophase band, a cortical array of microtubules which is thought to determine the exact site of cytokinesis. The cell plate starts to assemble in the center of the division plane in close association with two sets of polar spindle microtubules, the phragmoplast, and expands laterally toward the parental cell wall.

While the majority of plant cells divide by the mechanism described above, a few specialized types of cells make use of an alternative mechanism. In these cells the new cell walls originate as outgrowths of the preexisting walls and grow inward toward the center of the cell until they have achieved the partitioning of the cell (Farr, 1916, 1918). Perhaps the best studied example of this type of cell division is in the cytokinetic division of male meiosis in many species of dicots. In these species, the pollen mother cell (PMC)

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typically undergoes both meiotic nuclear divisions prior to the simultaneous division of the cell into four microspores by a centripetal division process. This division therefore differs in two fundamental ways from a conventional plant cytokinetic division: the cellular division is no longer tightly coupled to the nuclear division and the construction of the new cell wall occurs by a different mechanism.

Abundant genetic evidence has existed since shortly after the turn of the century that these two types of cell division were mechanistically distinct. Early work of Castetter (1925) and Moffett (1932) described the production of abnormally large pollen grains in lines of *Melilotus* and *Kniphofia*. Both of these papers describe the failure of cytokinesis following male meiosis, leading to the production of multinucleate microspores and oversize pollen grains in plants which showed no obvious defects in normal development. Failure of male meiotic cytokinesis due to a single gene mutation has also been described in maize (Beadle, 1932), soybean (Albertson and Palmer, 1979), and alfalfa (McCoy and Smith, 1983). Although some of these mutations do have effects on other specific types of cells (most notably female meiosis and embryo sac development), none of them cause defects in the normal process of cytokinesis by which plant cells divide. In contrast to this, mutations in the *KNOLLE* (*KN*) gene of *Arabidopsis* disrupt the normal cytokinetic process of somatic cells and result in embryonic defects (Lukowitz *et al.,* 1996). These mutations have no gametophytic effects on either pollen or embryo sac development, but since homozygous *kn* plants die as seedlings it is not possible to determine if the product of the *KN* gene is required sporophytically for male or female meiosis.

Interestingly, the phenotypic defect of these various mutations in different species are quite distinct. In the meiotic divisions leading to the production of giant pollen observed in lines of *Kniphofia,* two distinct types of defects were observed (Moffett, 1932). In some PMCs the spindle apparently fails to segregate the chromosomes leaving the chromosomes scattered throughout the cell. This leads to the formation of many micronuclei in a large microspore. In the second type of defect, chromosomes segregated normally but the formation of walls dividing the PMC into a tetrad was either absent or incomplete. This led to the production of microspores which could contain one, two, or all four meiotic products. In some cases incomplete cytokinesis resulted in the production of cytoplasmically independent microspores which remained attached to one another as tetrads. Mutations in the *QUARTET* (*QRT1, QRT2*) genes of *Arabidopsis* produce similar attached tetrads of pollen grains (Preuss *et al.,* 1994). In his description of the *variable sterile* mutant of maize Beadle (1932) describes the same two types of defects. In contrast to these two examples, mutations in the ms_1 gene of soybean (Albertson and Palmer, 1979) and the *jp* gene in alfalfa (McCoy and Smith, 1983) appear to produce a complete failure of cytokinesis following male meiosis, but do not produce chromosome segregation abnormalities. In both of these cases the mutations described also produce defects in the development of the female gametophyte, unlike the first two examples (McCoy and Smith, 1983; Kennell and Horner, 1985). The ultimate fate of the multinucleate microspores that are produced is quite different in the two species, despite the similarities in the apparent primary defect. In soybean, the microspores continue through an aberrant developmental process leading to the collapse and degeneration of most of the pollen grains. Mature grains are frequently clumped together and fail to be released from the anther. In alfalfa, on the other hand, the four nuclei contained in the microspore undergo fusion after which the pollen proceeds normally through subsequent developmental stages. In some cases the nuclear fusion is incomplete leading to mature grains which contain more than one generative cell or tube nucleus. When more than one generative cell is present, both are capable of undergoing the second mitotic division to form sperm cells. Despite this apparently normal developmental process, the fertility of the tetraploid pollen grains produced by the *jp* mutant of alfalfa is extremely limited.

In this paper we describe the isolation of mutations in *Arabidopsis* which have a phenotype similar to those described for maize, soybean, and alfalfa. Interestingly, the phenotype of these mutations is distinct from each of those described above, and provides some insight into the cellular and subcellular mechanisms which control the development of the microspore into the mature pollen grain in *Arabidopsis.*

MATERIALS AND METHODS

Plant Strains, Growth Conditions, and Mutagenesis

The wild-type strain used was *Arabidopsis thaliana* (L.) Heynh. var. *Landsberg* (*erecta* mutant). TH154 is a temperature-sensitive male-sterile strain derived from *erecta* in which female development is normal (Schneitz *et al.,* 1995). Plants were grown under constant illumination at 25°C and 70% relative humidity as described previously (Hülskamp *et al.,* 1995b).

The *std-1* and *std-2* alleles were isolated from mutageneses with ethyl methanesulfonate (EMS). EMS treatment of seeds was performed as described previously (Robinson-Beers *et al.,* 1992). In the mutagenesis which yielded the *std-1* allele, seed from the M_1 generation was harvested in pools of approximately 500 M_1 plants and approximately 500 M_2 seeds were screened from each pool. A total of 30 such pools were screened to obtain the *std-1* mutation. In the mutagenesis which yielded the *std-2* allele, a single silique was harvested from each M_1 plant and the M_2 seed from this silique was screened as a single family. Approximately 15,000 M_1 families were screened in this second mutagenesis.

The *std-3* mutation was recovered following mutagenesis with γ-irradiation. Approximately 50,000 *Arabidopsis* seeds were preimbibed at 4°C for 4 days. These seeds were then dried for 24 hr in a growth chamber at 25°C and 70% relative humidity. The seeds were then subjected to 20 krad of γ-irradiation, suspended in water, and planted. Seeds from these M_1 plants were harvested as pools of 60 plants and approximately 500 M_2 plants from each of 300 pools were screened.

Genetic Mapping

Plants homozygous for each *std* allele were outcrossed to wild-type plants of the Columbia ecotype. F_1 plants were allowed to self-fertilize and F_2 generation was scored for homozygous *std* plants. DNA was prepared from the homozygous mutant plants using the miniprep protocol described by Edwards *et al.* (1991) and genetic mapping was carried out using PCR-based genetic markers based on both SSLP (Bell and Ecker, 1994) and CAPS (Konieczny and Ausubel, 1993) technologies. All three *std* alleles were mapped independently and demonstrated to map between the CAPS markers *GL1* and *NIT1* on the lower arm of chromosome 3 with an LOD score of greater than 3.0 using Mapmaker/EXP version 3.0 (Lander *et al.*, 1987). The map distances were calculated using Mapmaker and are based on data from all three alleles (30/340 recombinant chromosomes for *GL1;* 12/328 recombinant chromosomes for *NIT1*).

Phenotypic Characterization of the Pollen, Ovule, and Zygote Phenotypes

Pollen development was analyzed in DAPI and aniline blue-stained whole-mount preparations using a protocol modified from Regan and Moffatt (1990). Anthers were dissected on a slide and mounted in an aqueous solution of 1 μg/ml DAPI, 0.05% aniline blue, 5% DMSO, and 1% Tween 20. Samples were incubated at 4°C for up to 5 hr before inspection. Approximately 200 individual pollen grains from each *std* allele were examined to determine the distribution of numbers of sperm cells. Scanning electron microscopy studies of mature pollen were performed as described previously (Hülskamp *et al.*, 1995a). The ultrastructure of pollen at different developmental stages was analyzed by transmission electron microscopy. For transmission electron microscopy anthers were fixed and processed as described by Lukowitz *et al.* (1996). Ultrathin 60- to 90-nm sections were analyzed with a Phillips CM10 microscope. *In vitro* pollen germination experiments were performed as described by Azarov *et al.* (1990).

Ovule and zygote phenotypes were inspected in cleared whole-mount ovule preparations. Ovules were stained and processed as described by Schneitz *et al.* (1995). Aniline blue staining of megasporocyte cytokinesis was also as described in Schneitz *et al.* (1995).

Microscopy and Graphic Work

Preparations were examined using either a Zeiss Axiophot or Nikon Microphot-FXA microscope equipped for differential interference contrast (DIC) and epifluorescence. Photographs were taken on Kodak Ektachrome color slide films and scanned using a Nikon Coolscan slide scanner. All images were processed using Adobe Photoshop and figures were prepared with either Aldus Freehand or Deneba Canvas software.

RESULTS

Isolation and Genetic Characterization of stud Mutants

In order to examine the range of mutant phenotypes that can cause sterility in *A. thaliana,* several independent mutageneses were conducted. These screens yielded a large number of interesting mutations, but the present manuscript will focus on one class of these mutations: those which produce larger than normal pollen grains. Three independent mutations of this type were isolated, the first from a bulk M_2 population of EMS-treated seed, the second from a single line M_2 screen of EMS-treated seed, and the third from a small scale γ-ray mutagenesis (see Materials and Methods for details). Genetic crosses between these three mutations revealed that in every possible pairwise combination the mutations failed to complement one another providing preliminary evidence that they represent mutant alleles of the same gene. To confirm this finding each of the mutations was mapped using a combination of PCR-based genetic markers following outcrossing to a polymorphic wild-type strain (see Materials and Methods). Each of the mutations was mapped to a position on chromosome 3, 9.7 cM south of the CAPS marker *GL1* and 3.8 cM north of the CAPS marker *NIT1*. Because of the prominent, male-specific phenotype we have named this gene *STUD* (*STD*) and have designated these three mutant alleles *std-1, std-2,* and *std-3*. By genetic criteria all three *std* alleles behave as monogenic recessive mutations. F_1 plants resulting from outcrossing to wild-type plants are fully fertile, demonstrating that *std* is truly recessive. In the following F_2 generation the ratio of wild-type to mutant plants does not deviate significantly from the expected 3:1 segregation for any of three alleles (224:52 for *std-1;* 93:25 for *std-2;* 272:87 for *std-3*). The slight excess of wild-type plants seen in the segregation of *std-1* probably reflects the fact that these F_2 plants were scored for semisterility rather than scoring the pollen phenotype directly.

All three mutations described in this paper were originally isolated on the basis of their semisterility. Subsequent examination of the plants bearing the homozygous mutations under the dissecting scope revealed that the pollen grains produced were distinctly larger than wild-type pollen. This aspect of the phenotype can be seen most clearly by examining the pollen grains with the scanning electron microscope (SEM). As can be seen in Fig. 1, the structure of the mutant pollen grains from the three *std* alleles is similar, although not identical. While mutant pollen from all three alleles is clearly seen to be larger than wild-type pollen, the surface features of the mutant pollen grains can be seen to vary from one allele to another. Wild-type pollen grains have an obvious network of ridges when viewed by SEM, as can be seen in Fig. 1A. The majority of the pollen grains produced by each mutant plant also have this ridged structure (Figs. 1B, 1D, and 1E), although the spacing between the ridges appears to be reduced in pollen grains from the *std-3* allele (Fig. 1E). In addition, while wild-type pollen grains always appear to have a well-defined network of ridges (310/310 grains examined), two of the three *std* alleles produce pollen grains which show aberrations in this pattern. Eight percent of the pollen grains produced by *std-1* mutant plants (20/241 grains examined) showed a poorly defined rough surface in place of the ridge network (Fig. 1C), while 4% of *std-3* grains demonstrated a completely smooth phenotype (6/161 grains examined; Fig. 1F). In no

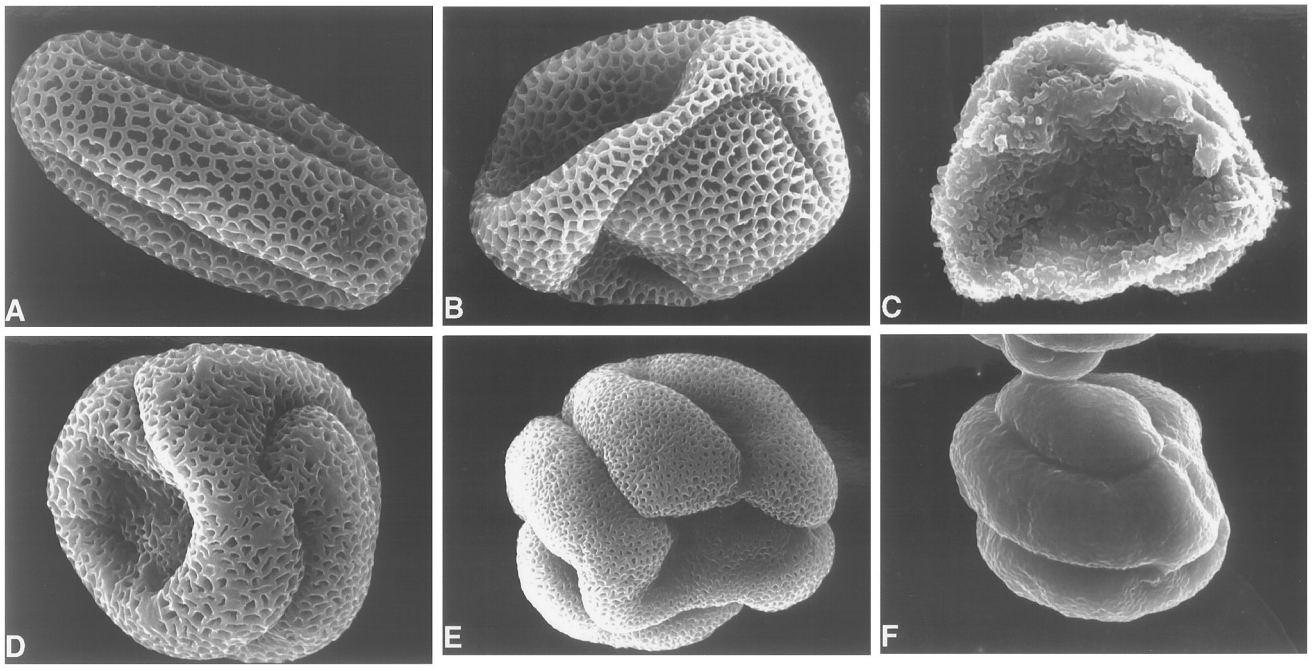

FIG. 1. Scanning electron micrographs of mature pollen grains from wild-type plants and *std* mutants. (A) Wild-type pollen grain. (B) Typical pollen grain from *std-1*. (C) Rough surfaced pollen grain from *std-1*. (D) Pollen grain from *std-2*. (E) Typical pollen grain from *std-3*. (F) Smooth-surfaced pollen grain from *std-3*.

case have we ever observed any wild-type pollen grains on plants homozygous for any *std* allele.

Given the dramatically altered structure of the pollen grains produced by *std* mutants, it is somewhat surprising that the mutations result only in semisterility. Since we have never seen any wild-type pollen grains produced by *std* mutant plants, this implies that the large *std* pollen grains must be capable of effecting fertilization, albeit with reduced efficiency. To examine this possibility, reciprocal crossing experiments were performed between *std-1* plants and wild-type plants (Table 1). When *std-1* pollen grains are used to pollinate wild-type plants, nearly 20% of the wild-type ovules are fertilized and begin seed development, thus demonstrating that *std* pollen grains are capable of delivering functional sperm cells to the embryo sac. Interestingly, the majority of these seeds later abort development (see below). The reciprocal experiment reveals that the presence of a homozygous *std-1* mutation appears to have no effect on the female reproductive system. When wild-type pollen grains are used to pollinate *std-1* mutant plants, fertility approaches 100% and no defects are seen during embryonic development.

Large Pollen Grains Result from a Defect in Male Meiotic Cytokinesis

In order to determine the nature of the developmental defect that leads to enlarged pollen grains, we examined microsporogenesis in *std* mutants in the light microscope. Anthers from various developmental stages were dissected open, stained with a combination of aniline blue and 4′, 6-diamidino-2-phenylindole (DAPI), and examined in a microscope equipped for epifluorescence. In wild-type pollen development the PMC undergoes two nuclear meiotic divi-

TABLE 1
Sterility in Reciprocal Crosses of *stud-1* and the Wild-Type Strain Landsberg *erecta*

	Normal seeds	Aborted seeds	Undeveloped ovules	Total ovules/seeds	Number of seed pods
stud-1 ♀ × *stud-1* ♂	105 (4%)	322 (11%)	2408 (85%)	2835	60
stud-1 ♀ × *er* ♂	1768 (96%)	—	71 (4%)	1839	37
TH154 ♀ × *stud-1* ♂	63 (4%)	219 (15%)	1195 (81%)	1457	31

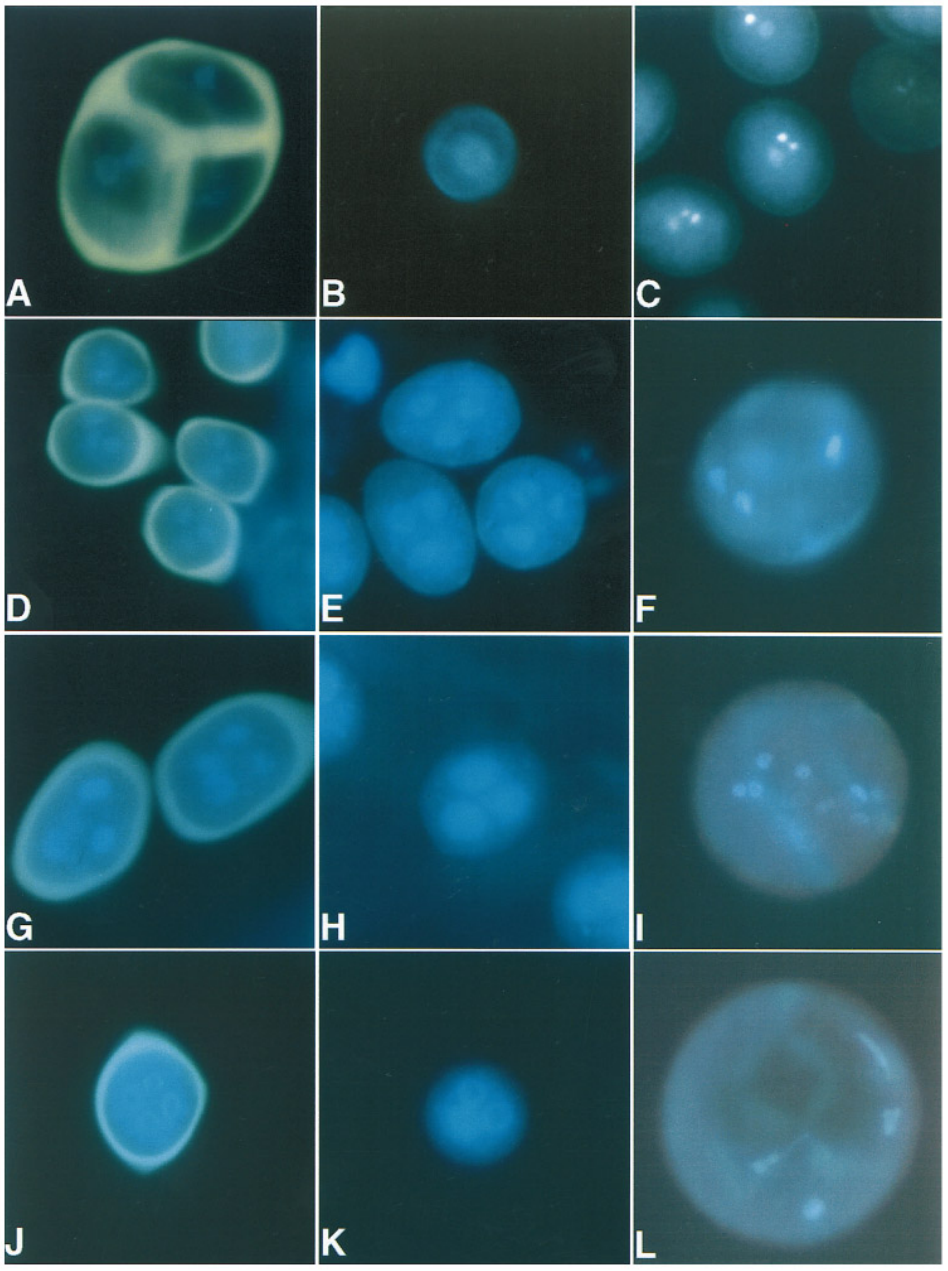

FIG. 2. Developmental stages of the male gametophyte as visualized in the light microscope after staining with aniline blue and DAPI. (A) Wild-type pollen mother cell at the tetrad stage. (B) Newly released wild-type microspore. (C) Mature wild-type pollen grain. (D) *std-1* pollen mother cell at the tetrad stage. (E) Newly released *std-1* microspore. (F) Mature *std-1* pollen grain. (G) *std-2* pollen mother cell at the tetrad stage. (H) Newly released *std-2* microspore. (I) Mature *std-2* pollen grain. (J) *std-3* pollen mother cell at the tetrad stage. (K) Newly released *std-3* microspore. (L) Mature *std-3* pollen grain.

sions before a simultaneous cytokinesis divides the four meiotic products. Following cytokinesis, callose deposition is found in all walls including the newly formed walls separating the four microspores (Fig. 2A). Shortly after this, the callose breaks down releasing the free microspores (Fig. 2B).The haploid microspores start to form a vacuole and undergo two mitotic divisions. Division I is asymmetric and produces a large vegetative and a small generative cell. In *Arabidopsis*, the generative cell divides again before pollen maturation to give rise to trinucleate pollen grains containing two sperm cells (Fig. 2C).

In *std* mutant PMCs, the two nuclear meiotic divisions appear to take place normally. A callose containing wall was seen to form around the periphery of the PMC at the

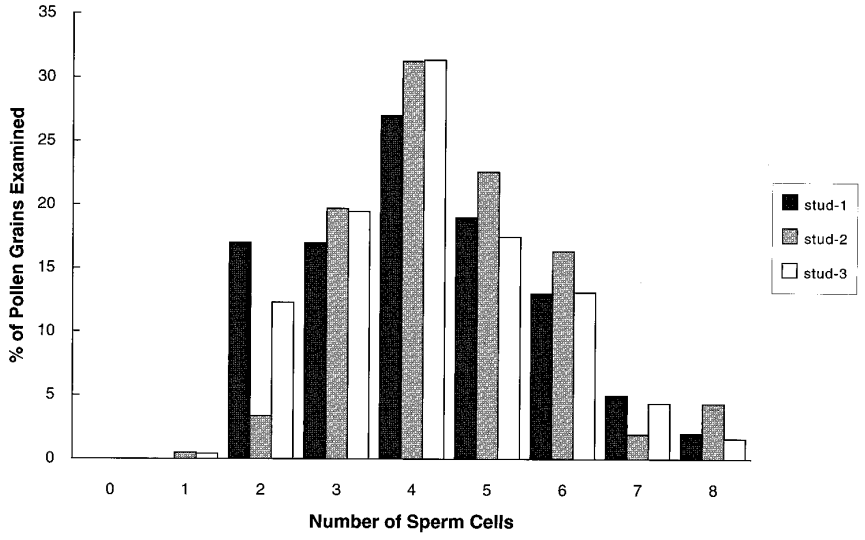

FIG. 3. Number of sperm cells found in *std* mutant pollen. The graph shows the percentage of pollen grains from each *std* allele which bear different numbers of sperm cells. 100% of wild-type pollen grains examined contained two sperm cells (179/179).

time when cytokinesis would normally be occurring, but callose containing walls between the nuclei were never observed (Figs. 2D, 2G, and 2J). Subsequently the ''microspores'' were released from the callose wall with four haploid nuclei in a common cytoplasm (Figs. 2E, 2H, and 2K). Further development and differentiation of the tetranucleate microspore appears to occur in a fairly normal manner, although it is usually incomplete. The multinucleate microspore undergoes asymmetric division to produce small intensely DAPI-staining generative cells. These generative cells also proceed through the second mitotic division at a low frequency resulting in mature pollen grains bearing a variable number of sperm cells.

In order to determine the distribution of sperm cell number in *std* mutant pollen grains, mature pollen grains isolated from plants homozygous for each of the three *std* alleles were stained with DAPI and viewed by epifluorescence microscopy. The number of intensely staining nuclei was counted and the data are presented in Fig. 3. Because the *std* pollen grains are relatively thick, it is possible that some nuclei were missed in this procedure and so these numbers should be considered as minimal estimates. As can be seen from the data presented in Fig. 3, the distribution of the numbers of sperm cells seen in the different alleles is quite similar. In every allele the range of sperm cell number was quite broad, being centered around four and ranging up to the eight sperm cells expected if both mitotic divisions took place normally. In no case were more than eight sperm cells observed. Although the number of pollen grains in which all possible mitotic divisions take place is low, the results clearly indicate that the separation of the haploid nuclear products into microspores is not a prerequisite for the asymmetric first mitotic division or the following symmetric division.

In order to examine the process of microsporogenesis in *std* mutants in more detail, anthers from various developmental stages were prepared for transmission electron microscopy (TEM) and the results are presented in Fig. 4. Examination of developing microspores from *std* mutants revealed a developmental pattern not dissimilar from wild-type microspores except for the presence of multiple nuclei within the *std* microspores. Multiple nuclei can be clearly seen in the newly released *std* microspore (Fig. 4D) as well as at the vacuolate stage (Fig. 4E). Asymmetric mitotic division takes place as in wild-type with multiple generative cells produced in the *std* microspore (Fig. 4F). These generative cells then undergo the usual process of engulfment (Fig. 4J) to produce mature pollen with a relatively normal ultrastructure (Fig. 4K). TEM observations of maturing *std* pollen grains did reveal abnormal internal cell walls at a very low frequency (2%; Figs. 4I, and 4L). Given the unusual folded appearance of many *std* pollen grains, these walls may be external walls that appear to be internal to the pollen grain due to the plane of the section.

Pollen Grains Produced by stud Plants Produce Single Pollen Tubes

Given the unusual character of the *std* pollen grains, we were interested in determining whether the mature pollen grain acted as a unit or as several independent entities. The data already presented make it clear that the tetranucleate microspores that are produced are capable of undergoing multiple asymmetric independent mitotic events to produce more than one generative cell from a single vegetative cell. In order to test whether the mature pollen grains also act in this way, wild-type and *std-1* pollen grains were germinated *in vitro* (Fig. 5). While the percentage of pollen

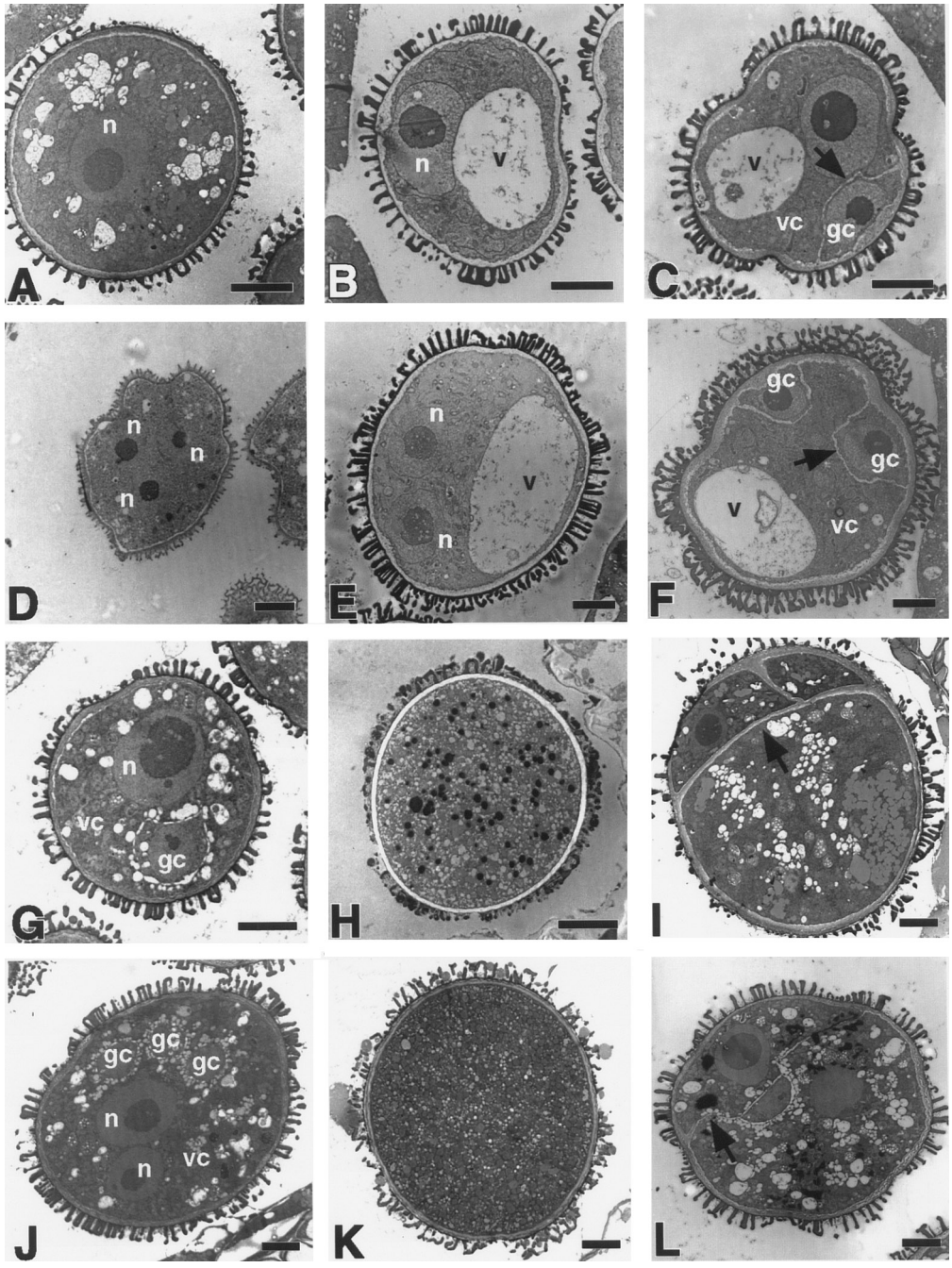

FIG. 4. Transmission electron micrographs of cross sections through anthers of *Arabidopsis thaliana*. Staging is as described in Owen and Makaroff (1995). (A) Wild-type released microspore I. (B) Wild-type vacuolate stage. (C) Wild-type bicellular pollen grain I, arrow indicates generative cell wall (fused with the intine of the vegetative cell). (D) *std-1* released microspore I. (E) *std-1* vacuolate stage. (F) *std-1* bicellular pollen grain I, arrow indicates generative cell wall (fused with the intine of the vegetative cell). (G) Wild-type bicellular pollen grain II. (H) Wild-type mature pollen grain. (I) *std-1* bicellular stage showing apparent internal cell wall (arrow). (J) *std-1* bicellular pollen grain II. (K) *std-1* mature pollen grain. (L) *std-1* bicellular stage showing apparent internal cell wall (arrow). Scale bar, 5 μm; gc, generative cell; n, nucleus; v, vacuole; vc, vegetative cell.

grains which germinated under these conditions was clearly different between wild-type (95%) and *std-1* (55%), all of the pollen grains which germinated produced only a single pollen tube regardless of genotype. Thus, although the *std* tetranucleate microspore is capable of undergoing multiple independent mitotic events, the mature *std-1* pol-

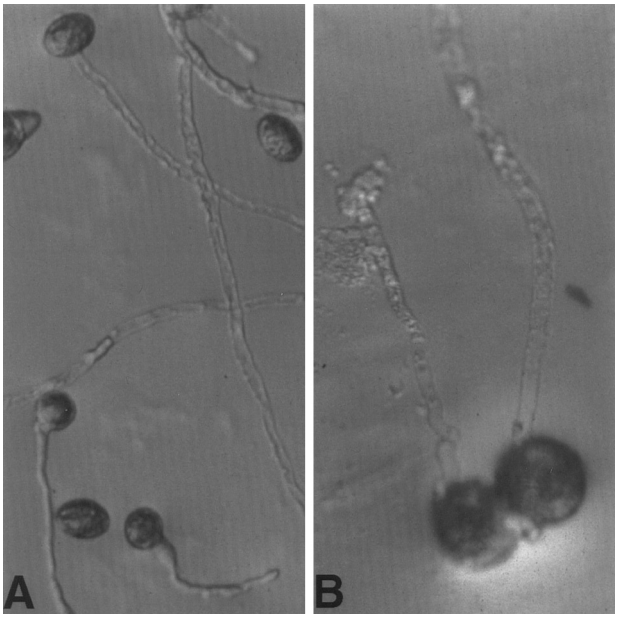

FIG. 5. *In vitro* germinated pollen grains. (A) Wild-type pollen. (B) Pollen from *std-1*.

len grain appears to act as a unit consisting of a single vegetative cell.

Cytokinesis Is Normal in Megasporogenesis

In order to test the possibility that *STD* might be required for meiosis-specific cytokinesis, we examined the progress of female meiosis in the light microscope. In *Arabidopsis* both meiotic nuclear divisions take place before the first cytokinesis. Cytokinesis can be followed in aniline blue-stained tissues to visualize the callose deposited in the newly formed cell walls. In *Arabidopsis* the tetrad can be found in either a multiplanar or linear array (Schneitz *et al.*, 1995). An analysis of megasporogenesis in *std-1* mutants revealed no principal deviations in cytokinesis from wild-type development, although callose deposition was often abnormally localized (Fig. 6). In an independent experiment we inspected more than 100 ovules at the tetrad stage in cleared whole-mount preparations. In these preparations all ovules contained clearly divided tetrads demonstrating that in *std* mutants cytokinesis is not affected during megasporogenesis (data not shown). An examination of mature ovules produced on *std-1* plants also revealed no differences from wild-type ovules (Fig. 6).

Fertilization with stud Pollen Leads to Embryo Abortion

The mutant *std* alleles described in this paper were originally isolated on the basis of their semisterility. Experiments described above demonstrated that the sterility was sporophytic, recessive, and limited to the male reproductive system. In order to better understand the mechanism of sterility in *std* mutants, we examined the maturing ovules from various crosses in cleared whole-mount preparations (Fig. 7). If *std* is used as the female parent (or both parents are wild-type), all of the embryos produced proceed through development normally. However, if *std* is used as the male parent (or both parents are *std*), many of the embryos produced fail to mature normally. Furthermore, the aborted embryos which are seen do not arrest at one specific stage of embryonic development but are distributed throughout a wide variety of developmental ages. Thus, although *std* pollen grains are clearly capable of effecting fertilization, many of the embryos resulting from those fertilization events are unable to develop normally. This developmental arrest is clearly not dependent only on the *std* genotype of the embryo since the reciprocal cross in which *std* plants are the female parent does not produce arrested embryos. Thus in a strictly formal genetic sense, *std* also behaves like a paternal effect mutation, conferring a high probability of embryo abortion on those zygotes which have homozygous *std* fathers. This is unlikely to be due to an imprinting effect since all offspring from *std* heterozygote fathers develop normally.

DISCUSSION

The first question that one wishes to address in the case of any new mutant phenotype is what is the nature of the

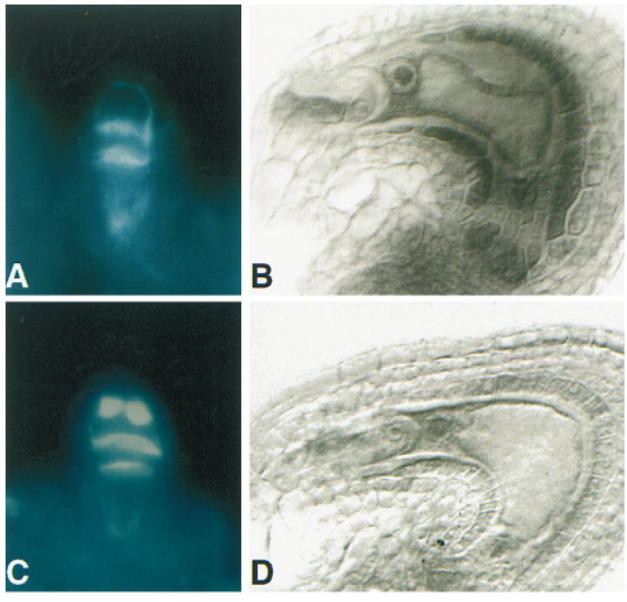

FIG. 6. Selected stages of female reproductive development in wild-type and *std-1* mutant plants. (A) Aniline blue-stained wild-type ovule at meiosis showing linear tetrad. (B) Cleared mature ovule from the male sterile line TH154 showing wild-type embryo sac structure. (C) Aniline blue-stained *std-1* ovule at meiosis showing linear tetrad. Note the abnormal callose deposition at the top of the tetrad. (D) Cleared mature ovule from *std-1* mutant plant showing normal embryo sac structure.

	Triangular Stage	Heart Stage	Mid-torpedo Stage	Mature Embryo Stage	Total
stud-1 ♀ x *stud-1* ♂	10%	46%	20%	24%	190
stud-1 ♀ x *er* ♂	-	-	-	100%	472
TH154 ♀ x *stud-1* ♂	9%	56%	23%	21%	134
er ♀ x *er* ♂	-	-	-	100%	520

FIG. 7. Stages of arrest seen in embryonic development of crosses involving *std-1*. Four crosses are depicted in which *std-1* represents both parents (line 1), *std-1* is the mother (line 2), *std-1* is the father (line 3), or both parents are wild-type (line 4). The images above the table illustrate the stages scored. The numbers in the body of the table reflect the percentages of embryos from each cross which were found to be arrested at a given stage. The total number of embryos scored from each type of cross can be found in the rightmost column.

defect? In the case of the *std* mutations that we have described it is clear that the most obvious defect is in their inability to undergo male meiotic cytokinesis. The defect conferred by this genetic lesion clearly does not extend to cytokinesis generally because we see no alteration in patterns of cell division in the somatic tissues of the plant. It is also clear that the *std* mutant phenotype does not extend to all cytokinetic events which follow multiple nuclear divisions, since the cell divisions following female meiosis as well as the mitotic nuclear divisions that lead to embryo sac formation appear to occur normally. Whether cellularization of the endosperm following its period of free nuclear division also occurs normally remains to be determined. Thus, the defect seen in *std* appears to be limited to male meiotic cytokinesis, implying that the gene product encoded by *STD* may be specifically required for this cytokinetic event. What distinguishes this cell division process from the others required for plant development? One possibility is the centripetal wall formation which has been found in pollen mother cell division in many dicot species. Unfortunately, we have been unable to assess any other centripetal wall synthesis systems in the *std* mutant background and so we do not know if *STD* is generally required for the division of cells by this type of mechanism. One possibility for the role of the *STD* gene product in male meiotic cytokinesis may be suggested by observation of tetrad formation in the female reproductive system. Although female tetrads are produced with high fidelity in *std* mutants, they frequently show aberrant deposition of callose (as determined by aniline blue staining) in locations which would never be observed in wild-type tetrads. It is possible, therefore, that *STD* is involved in the directed deposition of callose (as well as other wall materials) and that cytokinesis during male meiosis is dependent on the *STD* gene product to a greater degree than other types of cell divisions (including female meiosis). In this regard it is interesting that a gene required for somatic cell cytokinesis in *Arabidopsis*, *KNOLLE* (*KN*), encodes a protein with homology to syntaxins which are involved in vesicular trafficking (Lukowitz *et al.*, 1996). Whether the molecular role played by the *STD* gene product in male meiotic cytokinesis is related to that played by the product of the *KN* gene in normal cytokinesis must await the cloning of the *STD* gene.

An interesting aspect of the *std* phenotype is that, although it bears superficial similarity to the mutations found in other plant species which were described in the introduction, the phenotype is quite distinct from all of those examples. Mutant alleles of *STD* do not appear to cause any

serious defects in female meiosis or embryo sac formation, nor do they appear to result in chromosomal segregation abnormalities. This might reflect the fact that *std* is not truly homologous to these other mutations, but rather affects a different molecular process to produce a similar phenotype. In this regard it is interesting to note that in three independent mutageneses we recovered only three mutations producing large pollen grains and all of these ultimately turned out to be allelic. Although this does not completely exclude the possibility that there is another gene (the true homolog of the mutations seen in other species) having a mutant phenotype similar to *std,* it makes it unlikely.

The *std* mutants that we have isolated and characterized also reveal something of the developmental mechanisms which underlie the production of a mature pollen grain. Following its release from the tetrad, a wild-type microspore undergoes a very stereotypical asymmetric division. This division takes place after the nucleus moves to a position adjacent to the wall of the microspore and produces a small lens-shaped generative cell and a much larger vegetative cell (McCormick, 1993). The division exhibits a strong polarity not only in the sizes of the two products but also in the degree of condensation of the chromosomes, differences in which are already apparent prior to the cytokinetic division of the two cells (Terasaka and Niitsu, 1990; Brown and Lemmon, 1991). The generative cell is later engulfed such that it is entirely contained within the cytoplasm of the vegetative cell. Only then does the generative cell divide again to form the two sperm cells. In *std* mutant microspores much of this developmental process appears to be recapitulated. The first mitotic division shows the same asymmetric structure as in wild-type, with apparently normal generative cells forming along the outer microspore wall. The generative cells produced by *std* microspores appear to undergo the engulfment process normally. The data presented make clear that multiple independent mitotic divisions can take place within a single microspore, presumably each driven by one of the four haploid nuclei present within the microspore. Therefore we can say that this process is not dependent on cellular boundaries, but rather seems to be organized at some subcellular level. It is interesting in this regard to contrast the situation seen in multinucleate *std* microspores with the bicellular ''microspores'' produced by another *Arabidopsis* mutant, *sidecar pollen* (*scp;* Chen and McCormick, 1996). In *scp,* phenotypically normal microspores undergo a symmetric division to produce a microspore consisting of two equivalent cells. Only one of these cells then continues through the normal mitotic divisions of pollen development, the other remains as an extra vegetative cell within the mature pollen grain. This suggests the possible existence of a mechanism to ensure that only one cell undergoes mitotic division once two cells are present within the developing microspore. The presence of multiple mitotic divisions in the *std* microspores implies that such a mechanism is incapable of limiting cell division when multiple independent nuclei are present within a single cell.

An interesting question (which is not directly addressed by our data) then arises as to how the polarity of the mitotic divisions is maintained in the face of multiple asymmetric divisions taking place simultaneously. One can envision the cellular polarity required for the asymmetric division of the microspore as consisting of either a linear gradient across the cell or as a radial gradient from the center to the outside wall. If a linear gradient existed, all of the small generative cells in the *std* microspores would be expected to form on one side of the microspore, while in the case of a radial gradient they could be envisioned as occurring randomly anywhere around the periphery of the cell. Further examination of dividing *std* microspores should help to answer this question.

Mature pollen grains produced by *std* plants contain four vegetative nuclei and a variable number of sperm cells. While the average number of sperm cells seen in mature grains is well below the eight expected if all mitotic divisions proceeded normally, the presence of eight sperm cells in a reasonable proportion of the grains indicates that the second mitotic division can also take place in *std*'s unusual cellular environment. Furthermore, the presence of sperm cell numbers other than four and eight indicates that the generative cells which are produced in *std* appear to commit to a second cell division independently of one another rather than as a unified group. The fact that we have never seen more than eight sperm cells produced suggests that the mitotic processes following microspore release are still operating under normal developmental control, limiting each microspore nucleus to just two mitotic divisions.

In contrast to the mitotic cell division processes described above, mature *std* pollen appears to function as a unit. When *std* pollen grains are germinated *in vitro* single pollen tubes are produced, despite the presence of multiple vegetative nuclei. This indicates that the molecular processes which lead to the generation of a pollen tube are controlled at the cellular level, rather than being controlled in a subcellular manner as was true of the microspore mitoses. Once again it is interesting to compare the behavior of mature *std* pollen grains with that of pollen grains produced by *scp.* When *scp* pollen grains which contain two vegetative cells (only one of which contains sperm cells) are germinated *in vitro,* either of the two vegetative cells appears to be capable of growing a pollen tube, but two pollen tubes are never seen. This implies that production of the pollen tube is actually controlled at the level of the pollen grain (rather than by each vegetative cell), with some type of exclusion mechanism ensuring the production of only a single tube. Proper control of pollen tube development and sperm cell migration in *std* mutants probably also takes place *in vivo,* since *std* pollen grains are clearly able to function in effecting fertilization. The fact that fertilization by *std* pollen leads to a large fraction of abortive embryos, however, suggests that the functional male gametophytes produced from *std*'s tetranucleate microspores are seriously defective in some aspect of the fertilization process or a pollen-dependent function required for early embryogenesis. The nature of

this defect remains to be resolved and is an ongoing subject of investigation in our laboratory.

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